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(54) Title: MULTI REPORTER-LABELED NUCLEIC ACID PROBES

(57) Abstract

Nucleic acid probes, processes for using them, and processes for making them. The probes have reporter moieties such as fluorescent dyes covalently linked to phosphate atoms on the backbone of a single-stranded nucleic acid moiety.

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MULTIPLE REPORTER-LABELED NUCLEIC ACID PROBES

FIELD OF THE INVENTION

The inventions deal with nucleic acid hybridization probes, processes for using them, and processes for making them. The probes have reporter moieties such as fluorescent dyes covalently linked to phosphate atoms on the backbone of a single-stranded nucleic acid moiety.

BACKGROUND OF THE INVENTION

Probes made by coupling a nucleic single-stranded nucleic acid molecule to a fluorescent dye molecule, so as to create a molecule with both a nucleic acid moiety and a fluorescent dye moiety, are useful as probes for detecting gene sequences in nucleic acid hybridization reactions. (Hodges et al, Biochemistry 28, 261-267 (1989)). From the point of view of assay sensitivity it would be advantageous to use as many fluorescent dye moieties per probe molecule as possible. However, the dye moieties must be positioned on the probe molecule so as not to interfere with the hybridization capability of its nucleic acid moiety. Additionally, for in situ hybridization studies, the probe must be able to cross the cellular membrane and reach the intracellular nucleic acids. Therefore, the challenge of constructing a useful probe with multiple fluorescent dye moieties is a formidable one.

One of the present inventions involves the use of multi-fluor probes in hybridization assays, including in situ assays. In addition to the general benefit of increased sensitivity that such probes provide, there is the additional advantage that, for a given level of sensitivity to be reached, hybridization conditions less detrimental to cell integrity need to be used. As a result, the cells retain their structural integrity for a longer time, a consideration particularly important in the case of flow cytometry.

The ideas and procedures of the present inventions are not limited to fluorescent dyes, however. The same ideas and similar procedures can also be used with other types

of reporter moieties, such as those that participate in chemiluminescent reactions and those antigens that can be recognized by antibodies.

SUMMARY OF THE INVENTION

The inventions are nucleic acid probes, processes for using them, and processes for making them. Disclosed, for example, is that the fluorescent dye, fluorescein, can be modified to include a haloacetamido moiety which in turn can be covalently linked to multiple internucleoside phosphorothioate sites within a nucleic acid molecule. We have successfully used such multiple-label probes in hybridization experiments against nucleic acids extracted from cells. We have also used such probes successfully in in situ hybridization assays. As a result, the probes are useful as diagnostic tools for the detection of nucleic acids of pathogenic viruses and microorganisms. They are also useful in screening assays for genetic defects resulting from missing or detrimental nucleic acid sequences.

THE PRIOR ART

DeLong et al, Science 243, 1360-1363 (1989) disclosed the use of fluorescently labeled oligodeoxyribonucleotides to detect 16S ribosomal RNA in various eubacteria, eukaryotes and archaebacteria. The oligonucleotides had synthesized such that in the last stage an aminohexyl phosphate linker was attached to their 5' ends; subsequently the linker was linked to fluors such as fluorescein and rhodamine.

Fidanza et al, J. Amer. Chem. Soc. 111, 9117-9119 (1989) demonstrated that the dyes PROXYL and AEDANS can, via a halo-acetamido linkage, be introduced at single specific sites in a polynucleotide, the stated purpose being to create a molecule useful in studies involving protein bonding, resonance energy transfer, structural analyses and nucleic acid dynamics. Reactions were carried out in the presence of dimethylformamide and phosphate buffer at pH 6 and pH 8.

Agrawal et al, Nucleic Acids Res. 18, 5419-5423 (1990) attached two different fluorescent probes to the 3' and 5' ends of an oligonucleotide. One probe was monobromobimane attached to a phosphorothioate linkage between the two nucleosides.

The other probe was FITC attached to an amine linkage between the two nucleosides.

Hodges et al (1989) described a method for internally labeling phosphorothioate oligonucleotides with monobromobimane in a polyacrylamide gel.

Eshaghpour et al, Nucleic Acids Res. 7, 1485-1489 (1979) enzymatically attached 4-thiouridine to the 3' end of an oligonucleotide and then linked the sulfur atom to an alpha-haloacetamido dye derivative.

Connolly et al, Nucleic Acids Res. 13, 4485-4502 (1989) created oligonucleotides with S-triphenylmethyl moieties attached to the 5' phosphate moiety via a multicarbon chain. Ultimately, the free sulphydryl moiety was created the 5' end of the oligonucleotide and attached to 1,5-I-AEDANS.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 summarizes the reaction between an iodoacetamidofluorescein molecule and an internucleoside phosphorothioate sulfur atom.

Fig. 2 is an HPLC chromatogram of an oligonucleotide population that has been reacted with iodoacetamidofluorescein.

DETAILED DESCRIPTION

In one aspect, the invention is a process of forming a hybrid molecule between a nucleic acid probe to detect a nucleic acid target,

said probe comprising a single-stranded nucleic acid moiety and a plurality of reporter moieties such that each of said reporter moieties is covalently linked by means of a linker moiety to an intervening atom linked to an internucleoside phosphorus atom of said nucleic acid moiety;

said probe comprising a sequence of nucleosides complementary to a sequence of nucleosides in said target molecule,

said process comprising the steps of:

(1) incubating the probe and the target together in the same solution so as to allow them to hybridize to each other, and

(2) detecting the presence of target-bound probe molecules by a detection method that detects the presence of said reporter moieties.

The requirement that the probe comprise a sequence of nucleosides complementary to a sequence of nucleosides in the target molecule reflects the requirement that complementary is essential for the probe and the target to form a hybrid molecule (i.e., create a target-bound probe). The extent (length) of complementarity between the probe and the target will depend on the incubation conditions used for step (1). Many effective conditions for hybridization have been published in the literature. Usually, the length of the complementary sequence must be at least about 15 nucleosides for a stable hybrid to form.

An internucleoside phosphorous atom is one that is located between two nucleosides as opposed to being attached, at the 5' or 3' end of the oligonucleotide, to only one nucleoside.

A moiety is part of a molecule. For example, in Fig. 1, the nucleic acid moiety (the DNA strand), the acetamido moiety and the fluorescein moiety are each recognizable in the reaction product.

When a moiety links two molecular entities together, it may be referred to as a linker.

The term "dye" includes any molecule or molecular moiety that can be detected fluorimetrically or spectrophotometrically, especially though not necessarily in the visible range of wavelengths.

Preferred dyes are fluorescent dyes that absorb light in the visible range and emit light in the visible range.

The single-stranded nucleic acid moiety of the probe may be DNA or RNA.

Preferably the reporter moiety is a dye molecule; especially preferred is that the reporter moiety be a fluorescent dye moiety. A fluorescent dye can be detected in a flow cytometer or under a microscope fitted for detection of fluorescence.

The target nucleic acid molecule can be a purified nucleic acid molecule or one

located in a biological entity. A biological entity can be a cell or a virus. The cell or virus may be one that has been treated with a fixative.

A purified nucleic acid molecule is one that has been extracted from a cell or a virus, or has been synthesized in a cell-free system. Many procedures have been published for hybridizing a nucleic acid probe against a nucleic acid target that is either in solution or immobilized in single stranded form on a solid support such as a nitrocellulose filter or nylon. The hybridizations vary considerably, depending in part on the level of specificity desired. Some examples are the Southern Blot procedure (J. Mol. Biol., 98, 503-517 (1975)) for electrophoresed and immobilized DNA, the Northern Blot procedure (Seed, B., in Genetic Engineering: Principles and Methods, Setlow, J.K. and Hollaender, A., eds., 1982; P. S> Thomas, Proc. Natl. Acad. Sci. USA., 77: 5201 (1980)) and the use of stringent conditions with short oligomer probes, S. V. Suggs et al, Proc. Natl. Acad. Sci. USA, 78, 6613-6617 (1981)).

Viral RNA includes RNA that is genetic material, mRNA, and non-genetic material complementary to mRNA. Viral DNA includes genetic material (e.g., in "DNA viruses") and the product of reverse transcriptase or DNA copies thereof.

A viral nucleic acid can be part of a virus, in which case the virus may or may not be inside a cell. Alternatively, a viral nucleic acid target may not be part of a virus, but may be inside a cell.

During the hybridization reaction, the target nucleic acid must have to have a single-stranded region capable of reacting with the probe.

In order to form a stable double-stranded region between a probe and a target molecule, and do the hybridization under conditions where there is reasonable specificity of the hybridization reaction, it will generally be necessary to have a probe that is at least 15 nucleotides in length. When the target is a purified nucleic acid molecule, the probe molecule can generally be as large as desired, although generally it is preferred that the probe not be more than a million daltons.

Reporter moieties in the range 200 to 2000 daltons are preferred; those in the range 400 to 700 daltons are especially preferred.

When the reporter moiety is a fluorescent dye step (2) is performed by exposing

the target-bound probe to light at a wavelength that is absorbed by the dye, and detecting the light emitted by the dye moiety.

An example of a reporter group that can be detected by a nonfluorescent method is biotin, which can be detected on the basis of its ability to bind to second compound,streptavidin, which in turn can be linked to enzymes such as alkaline phosphatase or horse radish peroxidase that are detectable on the basis of their ability to react with a substrate. Moieties that participate in chemiluminescent reactions (e.g., (4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt), and moieties that react with antibodies are also among the possibilities for reporter moieties.

If as, in the case of a dye, the dye ring nearest the linker moiety will be separated from the phosphorous atom by at least two atoms (i.e., the "separation length" will be at least two atoms. At separation lengths of less than two atoms, the efficiency of constructing the probe is expected to decrease because of steric hindrance considerations. From the point of view of using the probe for hybridization, either where the target is a purified nucleic acid or one within a cell, there is no significant advantage to decreasing the separation length to less than four atoms.

An example of a separation length of 4 atoms is shown in Fig. 1. Also it can be seen in Fig. 1 that fluorescein is a dye moiety with 4 rings.

Where the reporter moiety is not a dye but nevertheless comprises a ring, the separation length is measured as in the case of a dye. Where the reporter group has no ring structure, the separation length is the separation length between the internucleoside phosphorus atom and the reporter moiety atom nearest that phosphorus atom.

It is clear from the foregoing that in counting the number of atoms between the dye ring and the phosphorus atom, one proceeds one atom at a time, proceeding from one atom to one covalently linked to it until one has covered, taking the shortest route, all the atoms between the ring and the phosphorus atom. For example, in Fig. 1, in the reaction product, the nitrogen atom would be numbered as atom number 1, the sulfur atom would be atom number 4, the two carbon atoms between the nitrogen and sulfur are numbers 2 and 3, respectively.

It is preferred that, as one proceeds along the shortest route between the dye ring

and the phosphorous atom, each atom is linked to the next one by a single covalent bond.

If the separation length is too great, one will decrease the chance that the probe can pass through the membrane pores through which it must travel in order to access targets in the cell nucleus. As a result, generally, separation of lengths 3 to 30 atoms are preferred. Separation lengths of 3 to 10 atoms are particularly preferred.

Although the presence of a dye linked to an internucleoside phosphorus did not eliminate the hybridization capability of an oligomer (See Examples below) it did decrease that capability. Additionally, if two dye moieties are too close to one another one would expect that there might be a quenching effect of one dye on the other. As a result, in order to minimize the potential anti-hybridization and quenching effects, it is preferred that the average number of nucleosides separating a dye-bound phosphorus atom from the next phosphorus atom along the oligonucleotide backbone be at least four atoms. More preferably, each phosphorus atom is separated from the next phosphorus atom along the oligonucleotide backbone by at least six atoms.

In one particularly preferred embodiment of a 30-mer (i.e., 30 nucleosides long), used to detect nuclear DNA, there are four internucleoside phosphorus atoms that are linked to a sulfur atom linked to a linker moiety linked to a dye moiety. Those four phosphorous atoms occur between nucleosides 1 and 2, between nucleosides 7 and 8, nucleosides 23 and 24 and nucleosides 29 and 30 (where the counting is done in a 5' to 3' direction).

In another particularly preferred embodiment of a 39-mer, useful to detect cytoplasmic RNA, there are also four internucleoside phosphorus atoms that are linked to a sulfur atom linked to the sulfur-linker-dye moiety. Those phosphorus atoms occur between nucleosides 1 and 2, 9 and 10, 30 and 31, and 38 and 39.

In both the above-noted 30-mer and 39-mer, it will be seen that there is a stretch of nucleosides in the middle of the probes such that there are no sulfur atoms linked to the phosphorous atoms. In the case of the 30-mer there is a 16-nucleoside long sulfur-free region. In the case of the 39-mer, there is a 21-nucleoside long sulfur-free region. These sulfur-free (and therefore sulfur-linker-dye free) regions are included because they

present an region whose hybridization ability is not decreased by a sulfur-linker-dye complex.

In the case of a single copy of a gene in a cell, particularly a human cell, it may be necessary to utilize a probe population with about eight hundred different 30-mers which, if laid end-to-end along a single target molecule in the order that they hybridize, would leave a 24,000 nucleoside-long sequence free of gaps along that target. With the probes and processes of this invention such a strategy is still a reasonable one. It is not necessary, for example, to use overlapping probes (the sequence at the 3' end of one probe being identical to the sequence at the 5' end of the other probe) the theory that part of each probe molecule will not hybridize because it has one or more phosphorus atoms linked to a sulfur-dye complex.

Dye molecules will typically have molecular weights in the range 400 to 700. However, larger dye molecules can be used; for example between 700 and 1500, can also be used.

For dyes on probes used to detect the targets in the cell nucleus (e.g., nuclear DNA), it is preferred that the length of the probe be less than about 40 nucleotides. If the probe had seven dye molecules, each with molecular weights between about 500 and 600, and seven linker moieties with a molecular weight similar to that of the acetamido moiety (about 60) and 40 nucleotides with an average molecular weight of about 345, then the molecular weight of the labeled probe would be about 20,000. A probe should normally be at least about 15 bases long for specific hybridization to take place.

For probes used to detect nucleic acids, especially RNA, in the cell cytoplasm, probes of not more than 200 nucleotides in length (molecular weight of not more than about 100,000) are preferred although larger oligonucleotides, for example, 1000 nucleotides long can be used.

The intervening atom between the linker moiety and the internucleoside phosphorous atom can, for example, be an oxygen atom, a sulfur atom, or a nitrogen atom. If the intervening atom is an oxygen atom, one has an internucleoside phosphotriester linkage. If the intervening atom is a sulfur atom, one has a an internucleoside phosphorothioate triester linkage. If the intervening atom is a nitrogen

atom, one has an internucleotide phosphoroamidate triester internucleoside linkage. (See Agrawal and Jamecnik, Nucleic Acids Research, 18, p.5419 (1990) for examples of the phosphorothioate triester and phosphoroamidate triester linkages. The triester linkage involve the diester linkage found along the backbone of naturally occurring nucleic acids plus a third ester linkage between the phosphorus atom and the intervening atom (the atom intervening between the phosphorus atom and the linker moiety).

If the intervening atom is a sulfur atom, then the linker moiety can be an acetamido moiety. In that case, if the dye is fluorescein, the number of atoms separating the dye molecule ring from the internucleoside phosphorus atom will be four (the nitrogen and two carbon atoms of the acetamido moiety plus the intervening sulfur atom).

If the intervening atom is a nitrogen atom, then the linker moiety can be an aminohexyl moiety. In that case, if the dye is fluorescein, the number of atoms separating the dye molecule ring nearest the linker from the internucleoside phosphorus atom will be eight, the nitrogen and six carbon atoms of the linker moiety plus the intervening nitrogen atom. If the dye molecule is fluorescein isothiocyanate, an additional two atoms, the nitrogen and carbon atoms contributed by the isothiocyanate moiety, will separate the dye molecule ring nearest the linker from the phosphorus atom.

It is understood that the hybrid molecule will be formed because the nucleic acid strand of the probe hybridizes to a nucleic acid strand of the target as a result of the fact that those two strands have a nucleoside sequence complementary to each other (e.g., the nucleoside's base, adenine complementary to either uracil or thymine in the other nucleoside, guanine complementary to cytosine), it not being necessary, however, that the entire nucleoside sequence of the probe be complementary to the entire nucleoside sequence of the target.

The fluorescent dye can be chosen as desired. Commonly used fluorescent dyes (noted with a convenient linker moiety) are 5-(2-(iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid, 5-iodoacetamidofluorescein, 6-iodoacetamidofluorescein, tetramethylrhodamine-5-iodoacetamide, tetramethylrhodamine-6-iodoacetamide, monobromobimane, erythrosin-5-iodoacetamide,

7-diethylamino-3-((4'-iodoacetylamino)phenyl)-4-methylcoumarin, 4'-((iodoacetylamino)methyl)fluorescein, erythrosin-5-iodoacetamide, biotin iodoacetamide, N-(1-pyrene)iodoacetate, biotin iodoacetamide, N-(1-pyrene)iodoacetate, N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole.

One of many alternatives to iodoacetamide is maleimide.

7-diethylamino-3-((4'-iodoacetylamino)phenyl)-4-methylcoumarin, and especially, 5-iodoacetamidofluorescein, 6-iodoacetamidofluorescein, tetramethylrhodamine-5-iodoacetamide, tetramethylrhodamine-6-iodoacetamide, and N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole.are preferred dyes for purposes of the present processes.

Monobromobiimine is a poor choice for in situ hybridization of eukaryotes, possibly because it does not efficiently pass through the cellular membranes.

It is important that the reporter moiety be stably linked to the nucleic acid probe moiety under conditions of the hybridization assay, give an adequate signal and, if it is a fluorescent dye, have usable excitation and emission wavelengths.

The probes used in the processes of the invention, including all preferred and specific embodiments of the processes, are also inventions. For example, an invention is a probe comprising a single-stranded oligonucleotide molecule and a plurality of reporter moieties such that each of said reporter moieties is covalently linked by means of a linker moiety to an intervening atom linked to an internucleoside phosphorus atom of the oligonucleotide. In a preferred embodiment, each of said dye moieties is covalently linked to an acetamido moiety that is covalently linked to the sulfur atom of an internucleoside phosphorothioate triester linkage.

A probe population may be constructed in which there are two subpopulations, one subpopulation consisting of probes of this invention having one type of dye (e.g., fluorescein) and the other subpopulation consisting of probes of this invention having a second type of dye (e.g., tetramethylrhodamine-5-(and-6)-iodoacetamide, where the two dyes are fluorimetrically distinguishable. Such a probe population can be used simultaneously to test for the presence and/or amount of two different nucleoside sequences in a target population.

Kits useful for carrying out the processes of the invention are also inventions. Such a kit would comprise a probe of the present inventions and one more reagents for use in a solution for reacting said probe population with said biological entity so that a hybrid molecule can form between a molecule of the probe population and a nucleic acid molecule in the biological entity. In a particular embodiment, such a kit would comprise one or more reagents selected from the group, a fixative and a chaotropic agent. Preferred are the fixatives and chaotropic reagents identified in this application.

The hybridization assay may be done with fixed cells (or fixed viruses). Fixatives should provide good preservation of cellular morphology and preservation and accessibility of antigens, and high hybridization efficiency. Useful precipitation fixatives include ethanol, acetic acid, methanol, acetone and combinations thereof. Other useful fixatives will be obvious to one skilled in the art. Fixatives and hybridization of fixed cells, in general, are discussed in PCT international applications, WO 90/02173 and WO 90/02204 of Research Development Corp.

Simultaneously, the fixative may contain a compound which fixes the cellular components by cross-linking these materials together, for example, glutaraldehyde, paraformaldehyde or formaldehyde. The cross linking agents when used are preferably less than 10% (v/v). Cross-linking agents, while preserving ultrastructure, often reduce hybridization efficiency, however; they form networks trapping nucleic acids and antigens and rendering them inaccessible to probes and antibodies. Some also covalently modify nucleic acids preventing later hybrid formation.

The hybridization solution may, for example, include a chaotropic denaturing agent, a buffer, a pore forming agent, and a hybrid stabilizing agent.

The chaotropic denaturing agents (Robinson, D. W. and Grant, M. E. (1966) J. Biol. Chem. 241: 4030; Hamaguchi, K. and Geiduscheck, E. P. (1962) J. Am. Chem. Soc. 84: 1329) include formamide, urea, thiocyanate, guanidine, trichloroacetate, tetramethylamine perchlorate, and sodium iodide. Any buffer which maintains pH at least between 7.0 and 8.0 is preferred.

The pore forming agent is for instance, a detergent such as Brij 35, Brij 58, sodium dodecyl sulfate, CHAPS™ Triton X-100. Depending on the location of the

target biopolymer, the pore-forming agent is chosen to facilitate probe entry through plasma, or nuclear membranes or cellular compartmental structures. For instance, 0.05% Brij 35 or 0.1% Triton X-100 will permit probe entry through the plasma membrane but not the nuclear membrane. Alternatively, sodium desoxycholate will allow probes to traverse the nuclear membrane. Thus, in order to restrict hydridization to the cytoplasmic biopolymer targets, nuclear membrane pore-forming agents are avoided. Such selective subcellular localization contributes to the specificity and sensitivity of the assay by eliminating probe hybridization to complementary nuclear sequences when the target biopolymer is located in the cytoplasm. Agents other than detergents such as fixatives may serve this function.

Hybrid stabilizing agents such as salts of mono- and di-valent cations are included in the hybridization solution to promote formation of hydrogen bonds between complementary sequences of the probe and its target biopolymer. Preferably sodium chloride at a concentration from 0.15 M to 1 M is used. In order to prevent non-specific binding of nucleic acid probes, nucleic acids unrelated to the target biopolymers are added to the hybridization solution.

Many types of solid supports for cells may be utilized to practice the invention. Supports which may be utilized include, but are not limited to, glass, Scotch tape (3M), nylon, Gene Screen Plus (New England Nuclear) and nitrocellulose. Most preferably glass microscope slides are used. The use of these supports and the procedures for depositing specimens thereon will be obvious to those of skill in the art. The choice of support material will depend upon the procedure for visualization of cells and the quantitation procedure used. Some filter materials are not uniformly thick and, thus, shrinking and swelling during in situ hybridization procedures is not uniform. In addition, some supports which autofluoresce will interfere with the determination of low level fluorescence. Glass microscope slides are most preferable as a solid support since they have high signal-to-noise ratios and can be treated to better retain tissue.

Step (2) of the process of this invention, the step where the probe and the target are co-incubated so that they can hybridize to each other, can be done with the target either in cells (or viruses) in liquid suspension, in cells (or viruses) on slides or other solid

supports, or cells (or viruses) in tissue sections. Such hybridization procedures are well known in the art. They are, for example, described in more detail in PCT applications WO 90/02173 and WO 90/02204.

The target molecules can be in non-enveloped viruses or enveloped viruses (having an enveloping membrane such as a lipid-protein membrane).

The cells containing target molecules may be eukaryotic cells (e.g., human cells), prokaryotic cells (e.g., bacteria), plant cells, or any other type of cell. They can be simple eukaryotes such as yeast or be derived from complex eukaryotes such as humans.

The cells can come from solid tissue (e.g., nerves, muscle, heart, skin, lungs, kidneys, pancreas, spleen, lymph nodes, testes, cervix, and brain) or cells present in membranes lining various tracts, conduits and cavities (such as the gastrointestinal tract, urinary tract, vas deferens, uterine cavity, uterine tube, vagina, respiratory tract, nasal cavity, oral cavity, pharynx, larynx, trachea, bronchi and lungs) or cells in an organism's fluids (e.g., urine, stomach fluid, sputum, blood and lymph fluid) or stool.

The target cells may be bacteria or other prokaryotes. They can be simple eukaryotes such as yeast or be derived from more complex eukaryotes such as humans. They can be plant cells.

Nucleic acid probes can be used against a variety of nucleic acid targets, viral, prokaryotic, and eukaryotic. The target may be a DNA target such as a gene (e.g., oncogene), control element (e.g., promoter, repressor, or enhancer), or sequence coding for ribosomal RNA, transfer RNA, or RNase P. The target may be RNA such as mRNA, ribosomal RNA, RNase P, tRNA, a viral genome or complementary copy thereof. Additionally, the target may be a "nucleic acid amplification product," i.e., a nucleic acid molecule, either DNA or RNA, which is the result of introducing an enzyme or enzymes into the cell so that such enzymes will make an nucleic acid molecule complementary to one already present in the cell. For example, O. Bagasra et al, The New England Journal of Medicine, 326, pp. 1385-1391 (1992), have disclosed the use of the polymerase chain reaction (PCR) with intact cells such that the introduction of polymerase molecules into a cell resulted in additional nucleic acid molecules being formed, each a copy of one previously existing in the cell, though not necessarily existing before the introduction of

the enzymes.

In another aspect, the invention is a process of making a probe molecule of the invention, which process comprises the steps of incubating a single stranded nucleic acid molecule (DNA or RNA) with a haloacetamido-dye molecule in an aqueous (ie., at least partially water) solution between pH 6 and pH 9, said single stranded nucleic acid molecule comprising an internucleoside phosphorothioate triester linkage. It is preferred that the aqueous solution comprise a phosphate buffer or a Tris buffer. In a particular embodiment of the process, 20 to 60 percent dimethylformamide (DMF) is in the aqueous solution. It is also preferred that the process be carried out at a temperature in the range 10 °C to 50 °C.

The stability of the product depends on the storage conditions. If the pH of the solution containing the dye labeled probe was greater than 9, there was complete hydrolysis within 24 hours. It is preferred, for storage that the pH be in the range, 6 to 7.

In a further embodiment of the process of making the probe molecule, where the desired amount of acetamido-dye molecules has not been reached in the initial reaction, additional haloacetamido-dye is added to the reaction mixture and allowed to react with the oligonucleotides.

Phosphorothioate Oligonucleotide Labeling Procedure

The labeling procedure for attaching an iodoacetamido-fluorescein molecule to a phosphorothioate oligonucleotide consists of combining the ingredients and allowing them to react, and isolating the product. The reaction is summarized in Fig.1 for the case where the dye is fluorescein. With polysulfurized oligonucleotides, after the initial reaction, more acetamido-dye is added in a "spike", and the reaction is allowed to proceed further. Monosulfurized oligonucleotides are reacted once with the dye while polysulfurized oligonucleotides are reacted twice. The second reaction or, "spike," improves the labeling efficiency.

Iodoacetamido-fluorescein is intrinsically unstable in light, especially in solution. Therefore, when the iodoacetamido-fluorescein, dissolved in DMF, is combined with the

reaction buffer, the water in the buffer competes with the sulfur on the oligonucleotide for the iodoacetamido-fluorescein. This can result in a lower labeling efficiency due to the loss of available reactive dye.

In our invention, for polysulfurized oligonucleotides, there is an initial reaction, a spike is added, and the reaction is allowed to proceed further. Limiting the amount of reactable dye in the initial reaction is preferable; possibly that is because it minimizes the excess dye which can react with the water.

When it is desired to have merely two atoms between fluorescein and the phosphorus-linked sulfur, one can purchase a commercially available compound, and 5-bromomethylfluorescein. Linkers that allow between 5 and 20 atoms between the dye ring and the phosphorus atom are: 1-pyrenemethyliodoacetate (a 4-atom linker, not including the sulfur atom intervening between the linker and the internucleoside phosphorous atom), N-((2-(iodoacetoxy)ethyl-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (a 6-atom linker) and 5-(2-(((iodoacetyl)amino)ethyl)amino)napthalene-1-sulfonic acid (a 6-atom linker), and succinimidyl 6-(6-(((iodoacetyl)amino)hexanoyl)amino)hexanoate (16-atom linker. In the case of the compound, succinimidyl 6-(6-(((iodoacetyl)amino)hexanoyl)amino)hexanoate, one first links a dye molecule (which may, for example, have six more atoms to donate to the linker) via its NH_2 group to that compound and then one reacts the product with the sulfurized nucleic acid moiety.

Reagents can be purchased from any of a variety of sources including Aldrich Chemical Co., Milwaukee, Wisconsin, Sigma Chemical Co., St. Louis, Missouri, Molecular Probes, Inc., Eugene, Oregon, Clontech, Palo Alto, California, Kodak, Rochester, NY, and Spectrum Chemical Manufacturing Corp., Gardenea, California.

Specific probes

The NR probe was complementary to part of the plant-specific nitrogen reductase gene and had the following nucleoside sequence:
TACGCTCGATCCAGCTATCAGCCGT (SEQ ID NO:1) where the sequence is left-to-right in the 5'-to-3' direction.

The HYR-2 probe, specific for a sequence in the human Y chromosome had the sequence CTACATTCCCTTCCATTCCAATGAA (SEQ ID NO:2).

The 28S RNA, specific for a sequence in human 28S RNA had the sequence ATCAGAGTAGTGGTATTTCACCGGC (SEQ ID NO:3).

Additional useful reagents and solutions

Useful reagents and solutions for executing the inventions described herein include 0.0025% Evans Blue and/or 10% dodecyl alcohol in the solution analyzed cytofluorimetrically; 5% (v/v) Vitamin E in the hybridization cocktail used where the assay target is in a biological entity; about 8% DMSO (v/v) with about 5% or 10% squalane and about 5% or 10% pyrrolidinone in the hybridization cocktail when the target is in a biological entity; 5 μl of 1 M (1 molar) DTT and 5 μl of Proteinase K (1 mg/ml) solution are added to 100 μl of cocktail and the hybridization reactin is run, for example, at 42°C for 5 min, then at 95°C for 5 min, and then at 42°C for 2 min, when the target is in a biological entity; about 0.05% or 0.01% aurintricarboxylic acid in the hybridization compound when the target is a biological entity.

Where 30-mers are used, probes against both strands of a double-stranded target can be used, provided that the probes are "out-of phase" along the map of the target so that any probe is not complementary in base sequence to more than about 15 nucleotides of a probe to the other strand of the target. In that way, probes hybridize to the target and not to each other.

Example 1

Reaction of monosulfurized oligonucleotides in phosphate buffer

200 ug of dried oligonucleotide was dissolved in 100 ul of 50 mM phosphate buffer, pH 7.0, to form a first solution. Then one mg of iodoacetamido-fluorescein was combined with 100 ul of dry DMF (i.e., 100 percent DMF) in a second solution. The two solutions were mixed together and shaken overnight. This resulted in an oligonucleotide to iodoacetamido-fluorescein ratio of 1:5. After the overnight incubation, the labeled oligonucleotide was precipitated with ethanol and 3 M sodium acetate. This crude material was then loaded on to a PD-10 column to remove free dye. The desired

fractions were collected. The liquid phase was then removed under vacuum. The crude material was then purified with HPLC.

Example 2

Synthesis of polysulfurized oligonucleotides in phosphate buffer

200 ug of dried oligonucleotide were dissolved in 100 ul of 50 mM phosphate buffer pH 7.0, to form a first solution. One mg of iodoacetamido-fluorescein was combined with 100 ul of dry DMF to create a 200 ul reaction mixture. The two solutions were mixed together and shaken overnight. This resulted in an oligonucleotide to acetamido-fluorescein ratio of 1:5 in the reaction mixture. One mg of iodoacetamido-fluorescein was again combined with 100 ul of dry DMF and this 100 ul was combined with the 200 ul of reaction mixture. Another 100 ul of 50 mM phosphate buffer was added to the 400 ul of reaction mixture and the reaction was allowed to continue for another 6 hours. The product was isolated as in Example 1.

Example 3

Synthesis of monosulfurized oligonucleotides in Tris buffer

The same procedure is followed as in Example 1 except that the 50 mM phosphate buffer is replaced with 250 mM Tris buffer, pH 7.4.

Example 4

Synthesis of polysulfurized oligonucleotides in Tris Buffer

The same procedure is followed as in Example 2 except that 50 mM phosphate buffer is replaced with 250 mM Tris buffer.

Example 5

Measurement of labeling efficiency of reaction using Tris buffer

The efficiency of our labeling reaction for an oligonucleotide made according to Example 3 was determined using HPLC chromatography monitoring the eluate from the column at an absorbance wavelength of 254 nm. The HPLC elution profile is shown in Fig. 2. The peak at 7.39 represents the unlabeled oligonucleotide. The peak at 8.63 represents the (iodoacetamido-fluorescein)-labeled phosphorothioate oligonucleotide. The peak at 8.63 was verified as the fluorescently labeled material in an independent HPLC chromatographic experiment (results not shown here) of the same sample chromatographed for Fig. 2 but where the eluate was exposed to light at 480 nm and light emission was measured at 520 nm.

Table 1 was produced by a computer program (product of Waters) used to generate the chromatograms shown in Fig. 2.

Table 1

Peak#	Retention Time	Peak Start	Peak End	Peak Area
1	0.767	0.358	0.858	13788
2	0.967	0.858	1.625	279915
3	1.158	1.100	1.292	17109
4	6.875	2.817	6.917	-196092
5	7.192	6.917	7.283	923779
6	7.392	7.283	7.942	1849961
7	8.633	7.942	10.150	27633087
8	10.758	10.150	14.183	11624176
9	15.633	14.650	18.808	647243
10	17.867	17.650	18.592	26972

For Table 1, the computer program integrated areas under the peaks in Fig 2. The efficiency of the labeling reaction is determined by dividing the area under the labeled oligonucleotide peak by the sum of the areas under the labeled oligonucleotide peak and the unlabeled oligonucleotide peak. The areas under the labeled oligonucleotide peak and the unlabeled oligonucleotide peak are 27,633,087 and 1,849,961 respectively. In

other words, 93.73% of the phosphorothioate oligonucleotide had a fluorescent label attached and 6.27% did not. Therefore, the labeling reaction was about 94 efficient. (That number may be actually be closer to 87 percent, as the single fluorescein on the oligomer is expected to contribute to the absorbance.)

Example 6

Measurement of labeling efficiency of reaction in phosphate buffer

Chromatographic analysis similar to that of Example 5 led to a labeling efficiency of 90.8% for this phosphate buffer system.

Example 7

Effect on Hybridization of a Reporter Group Linked to an Internal Phosphate

A 25-mer specific for 28S RNA was made with a single sulfur atom either between nucleosides 24 and 25 (counting in the 5' to 3' direction) (the "end-labeled probe) or between nucleosides 12 and 13 (the "mid-labeled probe"). Each probe was tested for its ability to hybridize in situ to ribosomal RNA.

Preparation of Cells

The H9 cell line was used. Cultured cells were washed with nuclease-free Phosphate Buffered Saline (PBS; 0.136 M NaCl, 0.003 M KCl, 0.008 M $Na_2HPO_4.7H_2O$, 0.001 M KH_2PO_4) and placed in a single cell suspension at a concentration that resulted in clearly separated cells. The cells were spun down to a pellet and the supernatant drained off. The cells were resuspend in 40% ethanol, 50% PBS, and 10% glacial acetic acid and left

for 12-16 hours at 4°C. After fixation, the cells were spun out of solution and then washed once in 1X PBS and resuspended in 2X SSC. (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; 2 X SSC is 0.30 M NaCl, 0.030 M sodium citrate pH 7) The cells were be used immediately.

Hybridization

For the hybridization procedure, to pelleted cells was added 50 μl of a hybridization cocktail consisting of 30% formamide, 5X SSC, O.16M sodium phosphate buffer, pH 7.4, 1 μg/μl sheared DNA, 3% (v/v) Triton X-100 (alcohol derivative of polyoxylene ether; see Aldrich Chemical Co. catalogue, 1990-91), 5% PEG 4000 (polyethylene glycol), 25 mM DTT (dithiothreitol), 0.4 M guanidinium isothiocyanate, 15X Ficoll/PVP, and the probe added at a concentration of 2.5 μg/ml. In the foregoing, 500X Ficoll/PVP is 5 g of Ficoll type 400 (polysucrose 400,000 mol wt) plus 5 g of PVP (polyvinylpyrrolidone) dissolved in water to a total volume of 100 ml; 15X Ficoll/PVP indicates that 500X Ficoll/PVP has been diluted with water by a factor of 15/500.

Hybridizations were carried out at 42°C for 30 minutes.

Washing

Proper washing after the hybridization reaction is essential to eliminate background due to non-specific binding of probe. Post-hybridization the cells were placed in a 15 ml conical tube to which was added 10 ml of a wash solution, preheated to 42°C, consisting of 0.1X SSC, 0.4 M guanidinium isothiocyanate, and 0.1% Triton. The solution was agitated until the cells were a single cell suspension and then spun at 1000 X g for 10 minutes. The supernatant was removed and to the pellet was added 10 ml of a wash solution, preheated to 42°C, consisting of 0.1X SSC, 0.1% Triton. The solution was agitated until the cells were a single cell suspension. The cells were spun at 1000 X g for 10 minutes. The supernatant was removed and the cell pellet resuspended in 0.2 ml counterstain solution consisting of 0.0025% Evans Blue in 1X PBS.

Flow Cytometer Use and Interpretation

The cells were analyzed on a Profile II™ made by Coulter Instruments. The Instrument uses a 488 nm argon laser, a 525 nm band pass filter for FL1 and a 635nm band pass filter for the counterstain. For each sample analyzed the sample containing the negative probe was analyzed first and the quad-stats were set so that less than 0.01% of the cells fell in the upper-right quadrant. Next the sample analyzed with the positive probe was analyzed under the exact same parameters as the sample analyzed with the negative prob. Since the quad-stats were set correctly and the two samples had been handled identically, any number of cells (above 0.01%) that were recorded in the upper right quadrant were scored as positive.

Sequence of steps

Approximately 500,000 H9 cells were equally divided into two tubes and fixed as described above. For one of these sample aliquots was added a hybridization solution containing a positive probe (28S) and to the other a negative probe (NR), corresponding to the same size as the positive probe as in the list in the table above. Following hybridization and washing, flow cytometry was performed.

Results

The results showed that the signal intensity obtained with the mid-labeled probe was detectable but only about two-thirds as great as that obtained with the end-labeled probe.

Example 8

Effect of spacing out sulfur-linked dye molecules on a 50-mer

Except for the probes used, experimental details were as in Example 7.

50-mer oligodeoxyribonucleotides specific for 28S and NR, respectively, were labeled with fluorescein-acetamido moieties at internucleoside (between nucleosides) phosphorus-linked sulfur atoms.

The sequence of the 28S 50-mer was:

GCCTCACCGGGTCAGTGAAAAAACGATCAGAGTAGTGGTATTTCACCGGC (SEQ ID NO:4).

The sequence of the NR 50-mer was:

CGCCTCGGAGTTGAAGGGATGTTTCCCTGTGAGACGTACCATGGAAGGGT (SEQ ID NO:5).

The number of nucleosides separating successive sulfur atoms was varied from probe to probe. The amount of signal observed with a sulfur-to-sulfur spacing of 4 nucleosides was only about as great as that obtained with either 6, 8 or 10 nucleosides between sulfur atoms.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Multiple Reporter-labeled Nucleic Acid Probes

(iii) NUMBER OF SEQUENCES: 5

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/915,927
(B) FILING DATE: 17-JUL-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Elman, Gerry J
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(C) REFERENCE/DOCKET NUMBER: M19-011

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TACGCTCGAT CCAGCTATCA GCCGT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTACATTCCC TTCCATTCCA ATGAA 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCAGAGTAG TGGTATTTCA CCGGC 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCTCACCGG GTCAGTGAAA AAACGATCAG AGTAGTGGTA TTTCACCGGC 50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCTCGGAG TTGAAGGGAT GTTTCCCTGT GAGACGTACC ATGGAAGGGT 50

CLAIMS

Having thus described the invention, what is desired to protect by Letters Patent and hereby claim is:

1. A process of using a nucleic acid probe to detect a nucleic acid target,

said probe comprising a single-stranded nucleic acid moiety and a plurality of reporter moieties such that each of said reporter moieties is covalently linked by means of a linker moiety to an intervening atom linked to an internucleoside phosphorus atom of said nucleic acid moiety;

said probe comprising a sequence of nucleosides complementary to a sequence of nucleosides in said target;

said process comprising the steps of:

(1) incubating the probe and the target together in the same solution so as to allow them to hybridize to each other, and

(2) detecting the presence of target-bound probe molecules by a detection method that detects the presence of said reporter moieties.

2. A process of Claim 1 wherein the target is in a biological entity, wherein said biological entity is either a either cell or virus.

3. A process of Claim 2 wherein the biological entity is a cell.

4. A process of Claim 3 wherein the cell is a eukaryotic cell.

5. A process of Claim 4 wherein the eukaryotic cell is a human cell.

6. A process of Claim 3 wherein the cell is a prokaryotic cell.

7. A process of Claim 3 wherein the cell is a plant cell.

8. A process of Claim 3 wherein the cell is a fixed cell.

10. A process of Claim 2 wherein the biological entity is a virus.

11. A process of Claim 10 wherein the virus is a fixed virus.

12. A process of Claim 2 wherein the biological entity is suspended in solution and is not immobilized on a solid support.

13. A process of Claim 2 wherein the biological cell is immobilized on a solid support.

14. A process of Claim 2 wherein the biological cell is part of a tissue section.

15. A process of Claim 1 wherein the target is a purified nucleic acid molecule.

16. A process of Claim 15 wherein the target is immobilized on a solid support during step (2) of the process.

17. A process of Claim 1 wherein the probe comprises a single-stranded DNA moiety.

18. A process of Claim 1 where the probe population has molecules capable of reacting with both RNA and DNA sequence in the probe population.

19. A process of Claim 1 wherein each of the reporter moieties comprises a ring structure and the separation length between said ring structure and the internucleoside phosphorous atom is at least two atoms.

20. A process of Claim 19 wherein the separation length between said ring structure and the internucleoside phosphorous atom is between 3 and 30 atoms.

21. A process of Claim 1 wherein the average number of nucleosides separating two reporter moiety-linked nucleic acid phosphorus atoms is at least four.

22. A process of Claim 1 wherein the intervening atom is selected from the group, an oxygen atom, a sulfur atom, and a nitrogen atom.

23. A process of Claim 1 wherein the probe comprises a plurality of reporter moieties that are fluorescent dye moieties.

24. A process of Claim 23 wherein step (2) is performed by means of flow cytometry.

25. A process of Claim 23 wherein step (2) is performed by means of a microscope fitted for fluorimetric detection.

26. A process of Claim 23 wherein the fluorescent dye moieties are fluorescein moieties.

27. A process of Claim 23 wherein step (2) comprises subjecting the target-bound probe to light of wavelength corresponding to an absorption wavelength of the fluorescent dye and then detecting light emitted at an emission wavelength of the fluorescent dye.

28. A probe comprising a single-stranded oligonucleotide molecule and a plurality of reporter moieties such that each of said reporter moieties is covalently linked by means of a linker moiety to an intervening atom linked to an internucleoside phosphorus atom of the oligonucleotide.

29. A probe of Claim 28 wherein each reporter moiety in the plurality comprises a ring structure and the separation length between said ring structure and a nucleic acid phosphorous atom is at least two atoms.

30. A probe of Claim 28 wherein the average number of nucleosides separating two

reporter moiety-linked nucleic acid phosphorus atoms is at least four.

31. A process of incubating a single-stranded nucleic acid molecule with a haloacetamido-dye molecule in an aqueous solution at a pH in the range 6 to 9, said single-stranded nucleic acid molecule comprising an internucleoside phosphorothioate triester linkage.

32. A kit for detecting a nucleic acid molecule in a biological entity, said kit comprising a probe of Claim 28 and one more reagents for use in a solution for reacting said probe population with said biological entity so that a hybrid molecule can form between a molecule of the probe population and a nucleic acid molecule in the biological entity.

33. A kit of Claim 32 wherein the biological entity is a cell and the one or more reagents comprise a reagent selected from the group, a fixative and a chaotropic agent. (Preferred are the fixatives and chaotropic reagents identified in this application.)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06682

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68, 1/70; C12P 19/34
US CL :435/5, 6, 91
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, ISSUED 1991, HAREWOOD ET AL, "CELLULAR UPTAKE AND LOCALIZATION OF FLUORESCEIN-LABELED, 15-MER PHOSPHOROTHIOATE AND PHOSPHODIESTER OLIGONUCLEOTIDES", PAGE 35, SEE ENTIRE ARTICLE.	1-33
A,P	US, A, 5,166,195 (ECKER) 24 NOVEMBER 1992, SEE SUMMARY OF INVENTION AND CLAIMS 1-2.	1-33
Y	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, VOLUME 111, ISSUED 1989, FIDANZA ET AL, "INTRODUCTION OF REPORTER GROUPS AT SPECIFIC SITES IN DNA CONTAINING PHOSPHOROTHIOATE DIESTERS", PAGES 9117-9119, SEE PAGES 9117-9118.	1-20, 28-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 September 1993	Date of mailing of the international search report 12 OCT 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MARGARET PARR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06682

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, VOLUME 243, ISSUED MARCH 1989, DELONG ET AL, "PHYLOGENETIC STAINS: RIBOSOMAL RNA-BASED PROBES FOR THE IDENTIFICATION OF SINGLE CELLS", PAGES 1360-1363, SEE ENTIRE ARTICLE.	1-33
Y	BIOCHEMISTRY, VOLUME 261, ISSUED 1989, HODGES ET AL, "POST-ASSAY COVALENT LABELING OF PHOSPHOROTHIOATE-CONTAINING NUCLEIC ACIDS WITH MULTIPLE FLUORESCENT MARKERS", PAGES 261-267, SEE PAGES 261-267.	1-25